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13. ABSTRACT (Maximum 200 Words)

Progression of breast tumors is dependent on blood vessel infiltration to supply nutrients and remove wastes. Expression of the angiogenic molecule vascular endothelial growth factor (VEGF) is a critical component of normal and pathological tissue vascularization. ErbB2, an epidermal growth factor receptor family member whose overexpression in mammary tumors is correlated with poor patient prognosis, has been previously implicated as a positive modulator of VEGF expression. I utilized a normal mouse mammary cell line (HC11) transfected with ErbB2 expression vectors to study the effects of ErbB2 overexpression on VEGF regulation. I have identified the promoter regions through which ErbB2 overexpression regulates VEGF expression by completing a structure-function analysis of the VEGF promoter in cultured mammary cells that overexpress ErbB2. ErbB2-mediated upregulation of VEGF involves at least two distinct promoter elements. One previously identified as the hypoxia responsive element and the other being the basal promoter region of the VEGF gene consisting of two adjacent SP1 transcription factor binding sites.

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INTRODUCTION

ErbB2 is one of four known receptors of the epidermal growth factor (EGF) receptor family and its overexpression is correlated with aggressive breast cancer (Slamon et al., 1987; Slamon et al., 1989). One possible mechanism for the aggressiveness of these tumors is that ErbB2 overexpression upregulates the angiogenic molecule vascular endothelial growth factor (VEGF). VEGF expression stimulates tumor vascularization, which accelerates tumor progression by promoting tumor growth and providing a conduit for metastasis (Borgstrom et al., 1999). Using a cell culture model of ErbB2-overexpressing mammary cells, I have determined that ErbB2 overexpression transcriptionally upregulates VEGF through the basal promoter found at -160 to – 51 base pairs (bp) of the VEGF gene.

FINAL SUMMARY BODY

Task 1. Establishment and screening of ErbB2-overexpressing mammary cell lines.

COMPLETED – see Annual Summary 2002 and below

ErbB2 overexpression has been correlated with aggressive forms of cancer in vivo and VEGF upregulation in vitro (Petit et al., 1997). I have established a mouse mammary cell model system to test the hypothesis that ErbB2 overexpression upregulates VEGF transcription. I chose to utilize a mouse cell culture model of ErbB2 overexpressing mammary tumors because several mouse mammary lines are well characterized, whereas human breast cancer systems are less well defined and matched control cell lines are not available. In addition, our lab cloned novel mouse VEGF promoter sequences and I prefer to test their activity in a mouse mammary cell line. I have screened several mouse cell lines and found an optimal mammary line to use in the VEGF promoter analysis (described below).

I conducted my initial VEGF promoter analysis with NAFA cells, a mouse mammary cell line derived from spontaneous tumors generated in mice engineered to overexpress a constitutively active form of ErbB2 (Andrechek et al., 2000) (a gift of William Muller, McMaster University, Canada). Although these cells did upregulate the VEGF promoter, no matched cell line was available for use as a negative control. I therefore chose to overexpress ErbB2 in normal mouse mammary cells. The two normal mouse mammary cell lines I chose for analysis were NMuMG (ATCC) and HC11 (a gift from David Stern, Yale).

Originally, I had proposed to make stable mammary cell lines overexpressing ErbB2, however, after researching this option, I concluded that ErbB2 expression levels would be more reproducible if transiently expressed as opposed to stable expression. I obtained an ErbB2 expression vector and a matching empty vector containing only the viral promoter (gifts from William Muller, McMaster University, Canada).

The VEGF promoter analysis in the both mouse cell lines transiently transfected with empty vector or ErbB2 confirms previous observations that ErbB2 upregulates transcription. Overexpression of ErbB2 in HC11 cells had a more pronounced effect on VEGF expression. Consistent with this observation, western blot analysis of endogenous ErbB2 expression levels in both lines revealed that HC11 cells have lower endogenous ErbB2 expression than NMuMG

cells, which likely makes HC11 cells more sensitive to ErbB2 overexpression induced VEGF upregulation (data not shown).

Task 2. Determine the smallest region of the VEGF promoter necessary for ErbB2-induced VEGF expression.

COMPLETED – see Annual Summary 2003 and below

Previously I had demonstrated that ErbB2 overexpression transcriptionally upregulates the VEGF gene through three potential areas of the promoter. The distal-most area (- 8 kb to – 4.3 kb) of the VEGF promoter was eliminated because it did not consistently respond to ErbB2 overexpression in two different mammary cell systems. The ErbB2-responsive area found at -1217 to –772 base pairs was fully characterized in the course of my studies by another group (Laughner et al., 2001). Because the proximal-most 450 bp of the VEGF promoter responded robustly to ErbB2 overexpression and was previously uncharacterized, it became the focus of my efforts. I have completed mapping the ErbB2 responsive element of the proximal VEGF promoter and my results are described below referencing data from my manuscript submitted to ***Journal of Biological Chemistry*** (appendix).

To confirm that ErbB2 overexpression leads to endogenous VEGF upregulation we quantitatively determined total VEGF and the proportion of VEGF isoforms in HC11 cells with or without ErbB2 overexpression. Endogenous VEGF mRNA levels significantly increase upon ErbB2 overexpression in HC11 cells compared with VEGF levels in empty vector transfected cells ($p < 0.01$, data not shown). Although the increase in total VEGF mRNA is modest (20%), it is interesting that there is a change in the proportion of VEGF isoform levels with ErbB2 overexpression (Figure 3).

The VEGF gene encodes for three murine isoforms: VEGF 188, VEGF 164 and VEGF 120 (Ferrara et al., 1991). Although all isoforms are secreted into the extracellular matrix, their spatial distribution varies as a function of the number of heparan sulfate proteoglycan-binding (HB) motifs. VEGF 120 isoform, lacking HB motifs, is freely soluble (Park et al., 1993) and interestingly tumor cells that overexpress VEGF 120 are better able to recruit surrounding vasculature toward the tumor (Grunstein et al., 2000). Therefore, I was intrigued to see that VEGF 120 expression increases 40% with ErbB2 overexpression while the other two VEGF isoform levels remain unchanged (Figure 3).

ErbB2 is one of four EGF receptors, and ErbB2 can either homodimerize or heterodimerize with the other three family members, EGFR, ErbB3 and ErbB4. It has been speculated that specific dimer pairs affect distinct downstream signaling pathways (reviewed in (Alroy and Yarden, 1997)). For this reason we identified and quantitated the EGF receptors expressed in parental and ErbB2-overexpressing HC11 cells. HC11 cells were transiently transfected with empty vector, wild type ErbB2 or constitutively active ErbB2, and protein levels were assessed by Western blot (Figure 4A). As expected, transfection of HC11 cells with either wild type or constitutively active ErbB2 expression vectors increased ErbB2 protein levels. Both ErbB2 and ErbB3 protein levels increase by 50% in cells transfected with wild type ErbB2 and by nearly 100% in HC11 cells transfected with constitutively active ErbB2, as compared to levels in control cells. EGFR protein levels were not affected by ErbB2 overexpression (Figure

4B). ErbB4 protein is not expressed at detectable levels under any condition in HC11 cells (data not shown).

To map the proximal promoter region that mediates VEGF expression in response to ErbB2 overexpression, deletions of the proximal 0.45 kb VEGF promoter-luciferase construct were made by a PCR approach (described in detail within materials and methods of manuscript). VEGF promoter deletion constructs were cotransfected into HC11 cells with either empty vector or constitutively active ErbB2 expression vector, and assayed for luciferase activity. Transfection of 0.45 kb-luc, 0.35 kb-luc, 0.24 kb-luc and 0.16 kb-luc all demonstrated significant upregulation when co-transfected with the ErbB2 expression vector as compared with empty vector controls ($p < .0001$, Figure 6); however, deletion of the promoter region -160 to -51 abolished the ErbB2-overxpression induced upregulation ($p=0.43$, Figure 6). Furthermore, the promoter activity in response to the empty vector cotransfection was also significantly reduced ($p < .05$), suggesting the -160 to -51 region is not only important for ErbB2 upregulation but also for basal regulation of the VEGF gene.

To specifically determine which transcription factor controls VEGF expression within the core promoter, additional VEGF promoter deletions were made of the 0.16 kb-luc construct (Figure 7A). Sequence analysis of the VEGF promoter revealed that two SP1 binding sites were the most likely downstream targets of ErbB2 signaling. Therefore, VEGF promoter deletion constructs, either wild type or mutant for two adjacent SP1 binding sites, were cotransfected into HC11 cells with either control vector or constitutively active ErbB2 expression vector, and assayed for luciferase activity. Promoter activity of WT 0.08 kb-luc and MUT 0.08 kb-luc were compared with 0.45 kb-luc and 0.05 kb-luc as positive and negative controls, respectively. Whereas the WT 0.08 kb-luc construct responded to ErbB2 overexpression with a 60% increase in promoter activity similar to the 0.45 kb-luc construct ($P < .0001$), mutation of the SP1 binding sites in MUT 0.08 kb-luc abolished ErbB2 overexpression-induced VEGF upregulation ($P = .44$) (Figure 7B). Additionally, mutation of SP1 binding sites significantly reduced VEGF expression even in the absence of ErbB2 overexpression when compared with either 0.45 kb-luc or WT 0.08 kb-luc ($P < .0001$) (Figure 7B). As expected neither the 0.05 kb-luc nor promoterless constructs upregulate VEGF in response to ErbB2 overexpression ($P = .66$ and .85, respectively). Consistent with a role for SP1 in the regulation of VEGF, transfection of ErbB2 into HC11 cells led to increased SP1 protein levels (Figure 7C).

Task 3. Analyze ErbB2-overexpressing human tumor samples for VEGF overexpression and correlate with patient outcome.

NOT COMPLETED

It has been established that ErbB2 overexpression can upregulate VEGF in mammary cells, but very few studies have been done in vivo that determine what percentage of human mammary tumors overexpress both ErbB2 and VEGF (Anan et al., 1998). This is relevant because independently, both ErbB2 and VEGF overexpression have been linked to breast tumor aggressiveness (reviewed in (Locopo et al., 1998; Revillion et al., 1998)). Moreover, it is not known if co-overexpression of these two molecules has adverse clinical consequences. For these reasons, I had planned to determine what percentage of ErbB2-overexpressing mammary tumors also express high levels of VEGF.

Our lab potentially has access to between one and two thousand archival invasive breast tumor samples (Dr. Simon Powell, program director Dana Farber – Harvard Cancer Center Breast Cancer program, personal communication). However, based on previous studies only thirty percent of the tumor samples are likely to be positive for ErbB2 overexpression (reviewed in (Revillion et al., 1998)). Of the roughly 300 – 600 ErbB2 positive breast tumors, I wanted to choose 100 tumors of similar profile, based on other predictive markers such as node and hormone receptor status and detect VEGF expression in these tumors by immunohistochemisty on paraffin sections with an antibody to human VEGF using established protocols. Because these are precious samples, I was unable to get access to any of these samples. To attempt to circumvent the problem of attaining precious human tumor samples, I also contacted a clinical lab at Harvard Medical School that works on ErbB2 overexpressing breast cancer to try to get mRNA from ErbB2-overexpressing mammary tumors. I planned to perform VEGF real-time PCR to determine if VEGF was upregulated in ErbB2-overexpressing tumor samples over levels found in non-ErbB2-expressing human mammary tumors. I was unsuccessful at attaining tumor RNA samples as well.

KEY RESEARCH ACCOMPLISHMENTS (2001-2004)

- **I screened several mouse cell lines and determined that the normal mouse mammary cell line (HC11) was the optimal model cell line to use in my VEGF promoter analysis (Task 2).**

- **I have determined that endogenous VEGF 120 isoform is increased in ErbB2 transfected HC11 cells.**

- **I have determined that ErbB3 but not EGFR or ErbB4 are coordinately upregulated in ErbB2 transfected HC11 cells.**

- **I have determined that ErbB2 overexpression upregulates VEGF through two adjacent SP1 binding sites in the basal promoter region found at –80 bp to – 51 bp.**

- **I have a manuscript submitted to the *Journal of Biological Chemistry* that I have attached as appendix material.**

REPORTABLE OUTCOMES (2001-2004)

Biggs, R.M., Ng, Y-S. and D'Amore, P.A. (2001) Temporal and spatial regulation of VEGF during in vitro vascular development in embryonic stem (ES) cells. 2001 Keystone Symposia on Angiogenesis and Chronic Disease Keystone, CO April 24-29, 2001.

Robyn M. Biggs, Yin-Shan Ng and Patricia A. D'Amore. Overexpression of ErbB2 transcriptionally regulates the vascular endothelial growth factor (VEGF) gene through a novel promoter element. Department of Defense Breast Cancer Research Program Meeting. Orlando, FL September 25-28, 2002.

Yin-Shan Ng, Markus Ramsauer, **Robyn M.B. Loureiro,** and Patricia A. D'Amore. Identification of genes involved in VEGF-mediated vascular morphogenesis using embryonic stem cell-derived cystic embryoid bodies. In press, ***Laboratory Investigation.***

CONCLUSIONS

Independently ErbB2 and VEGF overexpression are correlated with breast tumor aggressiveness. My work and the work of others have established that ErbB2 overexpression is molecularly linked to VEGF upregulation. In the past year, I have mapped the ErbB2-responsive region to the basal promoter region (-160 to –51 bp) of the VEGF gene. Specifically, VEGF is regulated through two adjacent SP1 binding sites in the basal promoter. Since several other genes such as interleukin 1B, hepatocyte growth factor, p53 and von Hippel-Lindau factor (Gille et al., 1998; Mukhopadhyay et al., 1997; Tanaka et al., 2000; Zhang et al., 2000) modulate VEGF expression through this promoter region, therapies directed at inhibiting the VEGF expression through the basal promoter would be no more effective than current systemic VEGF inhibitors, which have serious side effects (Stopeck et al., 2002). However, another possible point of VEGF inhibition in mammary tumor cells is VEGF isoform regulation. I have found that ErbB2 overexpression can change the VEGF isoform profile in mammary cells, which could alter the potential of mammary tumors to recruit blood vessels, but it is not known how isoform expression is regulated. I believe that breast cancer treatments of the future will be individualized based on tumor expression profiles; however, before that happens more research is needed to link molecular expression with patient outcome.

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ErbB2 Overexpression in Mammary Cells Upregulates Vascular Endothelial Growth Factor through the Core Promoter*

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Running title: Transcriptional regulation of VEGF by ErbB2

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Summary

Progression of tumors is dependent on blood vessel ingrowth to supply nutrients and remove waste. Expression of the angiogenic molecule vascular endothelial growth factor (VEGF) is a critical regulator of normal and pathologic angiogenesis. ErbB2, an epidermal growth factor receptor family member whose overexpression in mammary tumors is correlated with poor patient prognosis, has been previously implicated as a positive modulator of VEGF expression. Mammary tumor cells isolated from mouse mammary tumor virus (MMTV)-ErbB2 transgenic mice (NAFA cells) and a normal mouse mammary cell line (HC11) transfected with ErbB2 expression vectors were used to study the effects of ErbB2 overexpression on VEGF regulation. ErbB2 overexpression led to an increase in VEGF mRNA in HC11 cells. In ErbB2-overexpressing HC11 cells ErbB3 levels were increased whereas EGFR levels were unchanged. Structure-function analysis of the VEGF promoter was used to identify the promoter regions through which ErbB2 overexpression regulates VEGF expression in cultured mammary cells. We conclude that ErbB2 overexpression-mediated upregulation of VEGF involves at least two distinct promoter elements, one previously identified as the hypoxia responsive element and the other the core promoter region (-161 to –51 bp). Within the core promoter, VEGF expression is specifically controlled via two adjacent SP1 binding sites (-80 to –60 bp).

Introduction

VEGF[1] (also known as VEGF-A or vascular permeability factor) is an endothelial mitogen and stimulator of angiogenesis (1). VEGF is essential for normal embryonic development. In fact, inactivation of one copy of the VEGF gene causes a haploinsufficient embryonic-lethal phenotype, suggesting that correct vascular development is contingent on a tight dose-dependent regulation of VEGF expression (2,3). VEGF is also critical for tumor angiogenesis. VEGF-deficient embryonic stem cell-derived tumors form significantly smaller teratomas than control cells, and blocking VEGF in experimental tumor models leads to suppression of tumor growth and reduced metastasis (3-7).

The murine VEGF gene encodes three alternatively-spliced isoforms that differ in their amino acid number and in their heparan sulfate binding capacity (8). Although all isoforms are secreted, their spatial distribution varies according to the number of heparan sulfate proteoglycan-binding motifs. The VEGF188 isoform contains two heparan sulfate proteoglycan-binding motifs and is the most spatially restricted, whereas VEGF120, lacking heparan sulfate proteoglycan-binding motifs, is freely diffusible. VEGF164, with only one heparan sulfate proteoglycan-binding motif, has an intermediate distribution pattern (9). The various VEGF isoforms are functionally different as demonstrated by several *in vivo* studies of tumor and developmental angiogenesis (10-13).

A variety of factors induce VEGF expression (14-16). Among them, signaling through ErbB2, a transmembrane receptor tyrosine kinase, has been shown to upregulate VEGF in mammary tumor cells (17-20). Approximately 30% of breast tumors overexpress ErbB2, and these mammary tumors are characterized by poor patient prognosis and reduced tumor responsiveness to conventional chemotherapy (21). ErbB2 is a member of the EGF receptor

family, which also includes EGFR (ErbB1), ErbB3 and ErbB4 (22-24). A subset of the EGF family of ligands, including EGF, transforming growth factor α, betacellulin, amphiregulin and heregulin-α and -β, transduce their signal through ErbB2 (25) by promoting heterodimerization with the other three ligand-binding EGF receptors (26). Receptor heterodimers containing ErbB2 are potent effectors of the EGF signaling pathway because they inhibit ligand-dependent EGF receptor degradation and increase the affinity for EGFR ligands (27-29). ErbB2 overexpression promotes the formation of ErbB2 receptor homodimers, which have the potential to signal ligand-independently. Increased signaling through ErbB2 hetero- or homodimers *in vivo* stimulates cell proliferation, facilitating tumor growth and metastasis (30).

In addition to increasing tumor cell proliferation rates, ErbB2 overexpression may also accelerate tumor progression by upregulating VEGF, thereby promoting tumor vascularization. This potential link between ErbB2 overexpression and VEGF upregulation has been established by data from both *in vitro* and *in vivo* experiments. Treatment of ErbB2-overexpressing mammary tumor cells *in vitro* with a mouse ErbB2-specific function-blocking antibody leads to a dose-dependent decrease in VEGF protein production (31). A similar neutralizing antibody directed at human ErbB2, known commercially as Herceptin, has anti-angiogenic properties against human mammary tumors implanted in mice (32).

Some heregulin β1-responsive VEGF promoter elements have been characterized in ErbB2 overexpressing mammary tumor cell lines (19,20,33). However, it was not known whether ErbB2 overexpression in the absence of exogenous heregulin β1 upregulates VEGF exclusively through the heregulin β1-responsive elements, or if other novel promoter sites are also involved. Therefore, we have studied the mechanisms by which ErbB2 overexpression, in the absence of exogenous ligand, regulates VEGF expression in a mouse mammary cell model.

We have found that ErbB2 overexpression transcriptionally upregulates VEGF in mouse mammary cells and that this regulation occurs through the VEGF proximal promoter. Additionally, we have found that this ErbB2-responsive VEGF promoter region corresponds to the core promoter, consisting of two adjacent SP1 binding sites, rather than an ErbB2-specific enhancer as previously speculated.

Experimental procedures

Cell Culture

NAFA mammary epithelial cells were isolated from spontaneously formed mammary tumors in transgenic mice expressing constitutively activated ErbB2 under the control of a mammary specific promoter (MMTV-ErbB2) (34). Cells were maintained in DMEM-H (Gibco/Invitrogen Corporation, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 233.6 μg/ml glutamine, 80 units/ml penicillin and 80 μg/ml streptomycin (GPS; Irvine Scientific, Santa Ana, CA). HC11 cells (provided by David Stern at Yale Medical School, New Haven, CT) were maintained as previously described (35). Cells were grown at 37°C and 5% CO_2 for all experiments. Cell counts were determined using a Z1 Particle Counter (Coulter Corporation, Miami, FL).

Northern Blot Analysis

NAFA cells (2×10^5) were allowed to attach overnight in DMEM-H/FBS followed by serum-free media for 24 h, after which the 0 h time point was collected and the remaining cells refed with DMEM-H alone, DMEM-H containing 10 ng/ml EGF (Sigma-Aldrich, Inc., St. Louis, MO) or DMEM-H containing 100 ng/ml heregulin β1 (NeoMarkers, Fremont, CA) for the indicated

times. Cell cultures were observed to be of equal confluence prior to RNA collection. Total cellular RNA was isolated from NAFA cell cultures with RNAzol B (Tel-Test, Inc., Friendswood, TX) following the manufacturer's directions. RNA for a positive control was obtained from embryonic stem cells that were differentiated as aggregates for 10 days. Total RNA (30 μg) for each condition was loaded and separated on a 1% agarose/2.2M formaldehyde gel. RNA was visualized using ethidium bromide staining and transferred to GeneScreen Plus nylon membrane (NEN Life Science Products, Inc., Boston, MA). RNA was fixed to membrane by baking, then prehybridized at 42°C in ULTRAhyb (Ambion, Inc., Austin, TX). Hybridization was performed at 42°C using ULTRAhyb buffer containing ≈1 x 10^6 cpm [^{32}P]-labeled probe/ml. Hybridized membrane was washed and exposed to a Kodak Storage Phosphorimager screen (BioRad, Hercules, CA). To verify consistent loading, the membrane was stripped and reprobed for GAPDH. Hybridization probes were VEGF164 or GAPDH cDNA fragments radiolabeled with random primer oligonucleotides (Rediprime II Random Prime Labelling System, Amersham). Quantitative densitometry of the phosphorimage was performed using Quantity One software (BioRad). VEGF densitometry was normalized against 18S rRNA and GAPDH with similar results. This experiment was repeated twice with similar results.

Real-time RT-PCR

Total RNA (2 μg), collected as described above, was reverse transcribed using Superscript first-strand synthesis kit for RT-PCR (Stratagene) following the manufacturer's instructions. The VEGF isoforms were quantified by real-time PCR on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR was performed using primer sequences described previously (36) but with SYBR green PCR mastermix (Applied

Biosystems) following the manufacturer's instructions. Mean values from triplicate samples were graphed. VEGF isoform PCR values for each sample were normalized to a standard curve of known plasmid concentrations for each VEGF isoform respectively. "Relative mRNA levels" is a measure of VEGF isoform expression based on the concentration (in ng) of VEGF isoform plasmid DNA multiplied by 10^4, which results in whole number that can be easily graphed. This experiment was repeated twice with similar results. Statistical analysis of the data was performed to determine significance using Student's t-Test.

VEGF ELISA

CM was removed from NAFA cells and supplemented with 0.1% Tween 20 and protease inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml phenylmethyl sulfonyl fluoride and 10 mM sodium fluoride; Sigma). The cells were washed with PBS and cell-associated material was collected into lysis buffer (20mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 1% deoxycholic acid, 0.5% sodium dodecylsulfate, 1% Nonidet P-40 and protease inhibitors). Protein was quantified by D_C protein assay (BioRad, Hercules, CA), and 5-10 μg protein was assayed for each sample. VEGF protein levels were determined using a mouse VEGF ELISA kit (R&D Systems), which can detect as little as 3pg/ml VEGF. The A_{450} for each sample was determined relative to a VEGF dilution curve made from an internal standard. Mean values from duplicate samples were graphed. This experiment was repeated twice with similar results.

Western Blot Analysis

Protein concentration of cell-associated material (isolated as for VEGF ELISA) for HC11 cells transfected with control vector, wild type ErbB2 or constitutively activated ErbB2 was determined with a D_C protein assay kit (BioRad) to ensure even loading. The samples were

loaded in triplicate sequentially (10 μg of total protein/lane), separated on 6% polyacrylamide gels, and transferred to nitrocellulose filters as previously described (37). Filters were cut into three so that EGFR family members could be detected simultaneously. Filters were blocked in 5% non-fat powdered milk, 0.1% Tween 20 in PBS for one h at room temperature. Individual filters were incubated with a polyclonal antibody to EGFR (1:200; 1005, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a polyclonal antibody to ErbB2 (1:1000; Upstate Biotechnology, Lake Placid, NY) or a monoclonal antibody to ErbB3 (1:1000; Upstate Biotechnology). Filters were washed four times with exchanging 0.1% Tween 20 in PBS (PBST), 0.5M NaCl in PBST, 0.5% Triton-X in PBST and PBST, 10 min each. The filters were incubated with the appropriate goat anti-rabbit antibody coupled to horseradish peroxidase (1:5000, Amersham Biosciences, Buckinghamshire, UK) or sheep anti-mouse coupled to horseradish peroxidase (1:2000, Amersham). Washes were repeated as described and immunoreactive bands were visualized using ECLPlus (Amersham) and detected on Hyper film (Amersham). The protein sizes of the bands shown in Figures 4 were the only immunoreactive bands on the gel, and protein sizes were confirmed by comparison with prestained protein standards (Invitrogen) loaded adjacent to samples (not shown). Immunoreacted blots were scanned with Adobe Photoshop and densitometry was conducted using NIH Image software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/nih-image/). Protein bands were quantitated by comparison with amido black-stained blots as a loading control using NIH Image software. The value for control vector was set to one.

Immunoprecipitation and western blot analysis of SP1 protein

HC11 cells were washed with PBS and cell-associated material was collected into immunoprecipitation lysis buffer (10mM $NaPO_4$, 150 mM NaCl, 5 mM EDTA, 1% triton X100, 1% BSA, and protease inhibitors). Protein was quantified by D_C protein assay (BioRad, Hercules, CA), and 500 µg protein of each sample was incubated with 50 µg of agarose bead-conjugated anti-SP1 antibody (rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Beads were spun down and rinsed twice with PBS before resuspension in 20 µL loading dye. The entire sample was loaded and separated on a 4-20% gradient gel, and transferred as described above. Blots were also blocked and washed as described above. Filters were incubated with a polyclonal antibody to SP1 (1:200; sc-59, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The filter was striped and reincubated with a monoclonal SP1 antibody (1:200; 554129, BD Biosciences Pharmingen, San Diego, CA). Washes, secondary incubation and detection were carried out as described above.

Construction of VEGF Promoter-Luciferase Plasmids

The 1.2 kb-luc, 0.77 kb-luc, 0.45 kb-luc and +0.12 kb-luc have been previously described (38). Deletions of 0.45 kb-luc were individually constructed by PCR amplification using the following primers: 0.45 kb-luc forward 5'-GGGGTACCGGAGGACGCGTGTTTCAA-3',

0.35 kb-luc forward 5'-GGGGTACCTCCTCATACGTTCCTGCC-3',

0.24 kb-luc forward 5'-GGGGTACCTCTCCCCTGATTCCCAAT-3',

0.16 kb-luc forward 5'-GGGGTACCCCCTGGTAAGGGGTTTAG-3',

0.05 kb-luc forward 5'-GGGGTACCGGGGTGGAGCTAGATTTC-3',

WT 0.08 kb-luc forward 5'-GGGGTACCGTCCCCGGGGCGGGTCTGG-3'

MUT 0.08 kb-luc forward 5'-GGGGTACCGTCCCCGGGATGGGTCTGGATGGGG-3' and the reverse primer, 5'-GA***AGATCT***TCTCTCTGACCGGTCTCT-3', shared by all deletions. The resulting PCR fragments contain the deletions of the VEGF promoter flanked by 5' Kpn I (underlined) and 3' Bgl II (bold italics) restriction sites. Promoter deletions were amplified by Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). PCR fragments were purified and a 5' dATP-overhang was created by adding Taq DNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN) and an excess of dATP. ATP-overhang PCR fragments were ligated into the pGEM-T Easy TA cloning vector (Promega, Madison, WI). After verifying correct sequence, promoter deletions were excised from pGEM-T Easy with Kpn I (New England Biolabs, Inc., Beverly, MA) and Bgl II (NEB) and ligated into Kpn I/Bgl II digested pGL2-basic luciferase reporter plasmid (Promega) using QuickLink DNA ligation kit (Sigma). The construction of wild type ErbB2 and constitutively-active ErbB2 expression vectors was described previously (39).

Transient Transfections and Luciferase Assays

For DNA transfection cells were grown in media without antibiotics, according to the manufacturer's instructions for Fugene (Roche). NAFA cells were plated at 2 x 10^5 cells/well in 6-well plates. The cells were cotransfected at 60-80% confluence using Fugene with 75 pM VEGF luciferase-reporter plasmid (construction described above), 7.5 pM thymidine kinase promoter- *R*luc (Promega) and pBluescript (Stratagene) to a total of 1.5 μg DNA/well. HC11 cells were cotransfected with 120 pM VEGF luciferase-reporter plasmid, 12 pM *R*luc and pBluescript up to a total of 1 μg DNA/well. Where appropriate, 1 μg of either control vector or ErbB2 expression vector was cotransfected into HC11 cells using Fugene. Fresh medium was

added immediately prior to transfection and left undisturbed until cell lysates were collected after 48 h incubation. NAFA cells were transfected in DMEM-H/FBS and HC11 cells were transfected in DMEM-H containing 2% FBS. Luciferase expression was detected using the Dual Luciferase Reporter Assay System (Promega) following manufacture's instructions with a Turner Luminometer (Turner Designs, Sunnyvale, CA). Readings for VEGF-Fluc and *R*luc readings were recorded. VEGF promoter activity, as detected by Fluc activity, was normalized against *R*luc readings and this ratio was divided by ratio of readings from promoterless luciferase to determine fold-change. Assays were performed three times in triplicate with similar results; one representative experiment is shown. Statistical analysis of the data was performed to determine significance using analysis of variance.

Results

EGF and heregulin β1 both increase VEGF expression in ErbB2-overexpressing mouse mammary cells. Stimulation of human mammary tumor cells with EGF or heregulin β1 leads to increased VEGF mRNA levels (17-19). To determine whether murine cells respond similarly, VEGF mRNA levels were measured in EGF and heregulin β1-treated NAFA cells, an ErbB2-overexpressing mouse mammary cell line derived from spontaneous tumors in MMTV-ErbB2 transgenic mice. VEGF expression was assayed over 24 h to determine the time course of response to EGF or heregulin β1-stimulation (Figure 1A). As expected, serum removal resulted in a progressive decrease in VEGF expression, with minimal VEGF levels noted at 24 h after serum withdrawal (Figure 1B). EGF and heregulin β1 treatment increased VEGF mRNA expression as early as 2 h with a maximal effect at 24 h, when EGF and heregulin β1 increased VEGF expression by 4- and 3-fold, respectively, compared to untreated controls.

We next tested whether increased VEGF mRNA expression induced by EGF ligands leads to increased VEGF protein levels. VEGF protein was measured in CM and cell lysates using ELISA. Because the ELISA antibody recognizes VEGF120 and VEGF164 protein isoform and may not effectively recognize VEGF188[2], these data likely underestimate the total amount of VEGF protein. Nonetheless, both EGF and heregulin β1 treatment led to increased VEGF protein levels in NAFA cells compared with untreated cells (Figure 2). EGF and heregulin β1 increased VEGF production in the CM by 67% and 93%, respectively (Figure 2A). Cell-associated material includes intracellular proteins and cell-surface bound protein, as well as proteins associated with the extracellular matrix. EGF and heregulin β1 increased cell-associated VEGF by 30% and 60%, respectively (Figure 2B).

ErbB2 overexpression increases endogenous VEGF levels, and alters VEGF isoform expression patterns. Since the tumorigenic NAFA cells respond to EGF and heregulin β1, we next tested whether ErbB2 overexpression has similar effects on VEGF levels in a non-tumorigenic mouse mammary cell line (HC11). HC11 cells did not make detectable levels of VEGF mRNA as assessed by Northern blot analysis (data not shown). Therefore, quantitative RT-PCR was used to measure VEGF mRNA in HC11 cells transiently transfected with either a control vector or a construct expressing constitutively-active ErbB2. Expression of constitutively-active ErbB2 in HC11 cells led to a modest (20%) but statistically significant (Student's t-Test, $P < 0.01$) increase in total VEGF mRNA levels as compared to control vector transfected cells (data not shown). Furthermore, there was a significant change in the relative proportion of VEGF isoforms in cells expressing constitutively-active ErbB2. Although VEGF164 and VEGF188 mRNA levels did not change, VEGF120 mRNA levels increased by

40% with overexpression of constitutively-active ErbB2 (Figure 3). Transient transfection of wild type ErbB2 into HC11 mammary cells showed a similar trend in VEGF mRNA increase and isoform switch but the changes were not statistically significant (data not shown).

ErbB2 overexpression increases levels of ErbB3 but not EGFR protein. ErbB2, one of four EGF receptors, can either homodimerize or heterodimerize with the other three family members, EGFR, ErbB3 and ErbB4. It has been speculated that specific dimer pairs affect distinct downstream signaling pathways (25). For this reason, we investigated the effect of ErbB2 overexpression on the levels of EGF receptors in HC11 cells. HC11 cells were transiently transfected with control vector, wild type ErbB2 or constitutively-active ErbB2, and levels of the various EGF receptor family members were assessed by Western blot analysis (Figure 4A). As expected, transfection of the cells with either wild type or constitutively-active ErbB2 expression vectors increased ErbB2 levels. ErbB2 and ErbB3 protein levels increased by 50% in cells transfected with wild type ErbB2 and by about 100% in HC11 cells transfected with constitutively active ErbB2, as compared to levels in control cells. EGFR protein levels were not affected by ErbB2 overexpression (Figure 4B). ErbB4 protein was not expressed at detectable levels under any condition in HC11 cells (data not shown).

VEGF transcription is regulated through two promoter regions in ErbB2-overexpressing mammary cells. To determine the transcriptional elements necessary for VEGF upregulation in ErbB2-overexpressing cells, VEGF promoter deletion analysis was carried out in NAFA cells. NAFA cell lysates were analyzed for VEGF promoter activity after transient transfection with each of the four VEGF promoter-luciferase constructs or the pGL2-basic control vector. The

1.2 kb-luc construct contains 1.2 kb of the VEGF promoter and 370 bp of 5' UTR driving firefly luciferase reporter gene expression. All other constructs contain the same 370 bp of VEGF 5'UTR, but have 5' deletions of promoter regions and are named according to the length of intact VEGF promoter. The promoterless construct (pGL2-basic) contains only the firefly luciferase reporter gene. The +0.12 kb-luc construct contains no promoter sequence, and is composed of the VEGF 5' UTR region from +125 bp to +370 bp. A 10-fold increase in VEGF promoter activity was seen with the 1.2 kb-luc construct compared to 0.77 kb-luc and 0.45 kb-luc constructs ($P < 0.0001$), suggesting that the -1.2 kb to -770 bp region contains regulatory elements important for VEGF expression in cells overexpressing ErbB2 (Figure 5). Activity of the 0.45 kb-luc VEGF promoter construct was 30-fold greater than the promoterless construct and the +0.12 kb-luc, VEGF UTR-only construct ($P < 0.0001$), indicating that the 450 bp of the VEGF promoter proximal to the transcription start site likely contains an ErbB2-responsive regulator element. There was no significant difference between VEGF promoter activity of the 0.45 kb-luc and 0.77 kb-luc constructs; thus the promoter region -770 to -450 does not appear to contain regulatory regions important for VEGF expression in NAFA cells. As expected, the two promoterless luciferase vectors, +0.12 kb-luc and pGL2-basic, had no activity in ErbB2-overexpressing mammary cells. To further demonstrate that these effects are specifically caused by ErbB2-overexpression, these four VEGF promoter luciferase constructs were tested in HC11 cells. Cotransfection of the VEGF promoter luciferase constructs with an ErbB2 expression vector in HC11 cells revealed that VEGF expression was regulated by the same two promoter regions as in NAFA cells (data not shown and Figure 6).

ErbB2 upregulates VEGF transcription through the core promoter. The ErbB2-responsive element contained within the distal VEGF promoter has been previously described (-1.2 to –770, (40). To map the proximal promoter region that mediates VEGF expression in response to ErbB2 overexpression, deletions of the proximal 0.45 kb VEGF promoter-luciferase construct were made. VEGF promoter deletion constructs were cotransfected into HC11 cells with either control vector or constitutively active ErbB2 expression vector, and assayed for luciferase activity. Transfection of 0.45 kb-luc, 0.35 kb-luc, 0.24 kb-luc and 0.16 kb-luc constructs all demonstrated significant upregulation when co-transfected with the ErbB2 expression vector as compared with vector controls ($P < 0.0001$); however, deletion of the promoter region –160 bp to -51 bp abolished the ErbB2-induced upregulation ($P = 0.43$, Figure 6). Furthermore, the VEGF promoter activity in response to the control vector cotransfection was also significantly reduced ($P < 0.05$), indicating the -160 bp to -51 bp region is important not only for ErbB2 upregulation but also for basal regulation of the VEGF gene. Although the 0.05 kb-luc construct shows a 30-fold increase over the promoterless construct, the activity of the 0.05 kb-luc construct in the absence of ErbB2 is not significantly different than that of the promoterless construct ($P = 0.49$), which further suggests that the VEGF core promoter has been deleted in the 0.05 kb-luc construct.

Two adjacent SP1 binding sites within the core promoter control basal and ErbB2-induced VEGF expression. To specifically determine which transcription factor controls VEGF expression within the core promoter, additional VEGF promoter deletions were made of the 0.16 kb-luc construct (Figure 7A). Sequence analysis of the VEGF promoter revealed that two SP1 binding sites were the most likely downstream targets of ErbB2 signaling. Therefore,

VEGF promoter deletion constructs, either wild type or mutant for two adjacent SP1 binding sites, were cotransfected into HC11 cells with either control vector or constitutively active ErbB2 expression vector, and assayed for luciferase activity. Promoter activity of WT 0.08 kb-luc and MUT 0.08 kb-luc were compared with 0.45 kb-luc and 0.05 kb-luc as positive and negative controls, respectively. Whereas the WT 0.08 kb-luc construct responded to ErbB2 overexpression with a 60% increase in promoter activity similar to the 0.45 kb-luc construct ($P < .0001$), mutation of the SP1 binding sites in MUT 0.08 kb-luc abolished ErbB2 overexpression-induced VEGF upregulation ($P = .44$) (Figure 7B). Additionally, mutation of SP1 binding sites significantly reduced VEGF expression even in the absence of ErbB2 overexpression when compared with either 0.45 kb-luc or WT 0.08 kb-luc ($P < .0001$) (Figure 7B). As expected neither the 0.05 kb-luc nor promoterless constructs upregulate VEGF in response to ErbB2 overexpression ($P = .66$ and .85, respectively). Consistent with a role for SP1 in the regulation of VEGF, transfection of ErbB2 into HC11 cells led to increased SP1 protein levels (Figure 7C).

Discussion

Our results demonstrate that tumorigenic ErbB2-overexpressing mouse mammary cells respond to EGF and heregulin β1 in a manner similar to that previously reported for human tumor cell lines (17-19). We have shown that ErbB2 overexpression in the absence of EGF family ligands upregulates endogenous VEGF and alters the profile of VEGF isoforms toward the more freely soluble VEGF120. Additionally, we have demonstrated that ErbB2-overexpression upregulates VEGF through at least two promoter regions, the HRE, as previously described (40), and the core promoter through SP1 binding sites.

EGF and heregulin-β1 can upregulate VEGF mRNA and protein in the absence of ErbB2 overexpression (17-19). However, EGF or heregulin β1 stimulation of ErbB2-overexpressing cells enhances VEGF upregulation (19,20). Previous studies have relied on the use of human tumor cell lines whose genetic background cannot be sufficiently screened to rule out the effects of other mutations. Transgenic mice engineered to overexpress constitutively active ErbB2 under the MMTV promoter develop spontaneous mammary tumors at a young age (34). Using mammary tumor cells isolated from these transgenic mice, we have demonstrated that EGF and heregulin β1 upregulate VEGF mRNA and protein in mouse mammary tumor cells overexpressing ErbB2.

To confirm that ErbB2 overexpression leads to upregulation of endogenous VEGF we determined the levels of total VEGF and the proportion of VEGF isoforms in HC11 cells with or without ErbB2 overexpression. Unexpectedly, a switch in the proportion of VEGF isoforms was observed with ErbB2 overexpression. Regardless of the condition, VEGF188 levels made up less than 1% of total VEGF. However, a significant increase in VEGF120 levels was seen with ErbB2 overexpression. Although the role of individual VEGF isoforms has not been completely elucidated, they have been shown to be functionally distinct. For example, we have shown that mice engineered to express only VEGF120 die perinatally (10). Of more relevance to the current discussion, tumor cells that overexpress VEGF120 are better able to recruit vasculature toward the tumor (11). This is not surprising since VEGF120 is the most soluble isoform and thus would diffuse most efficiently, creating a chemotactic gradient to promote endothelial cell migration towards and into the tumor.

In biopsies of human breast carcinoma, there is a correlation between ErbB2 overexpression and overexpression of both EGFR and ErbB3 (41-43); however, there is no

agreement on the role of ErbB4 expression and its correlation with ErbB2 overexpression in human tumors (44,45). Thus, it is relevant to know which EGF receptor family members increase in mammary cells overexpressing ErbB2 compared with non-overexpressing cells. It has been previously shown that EGFR and ErbB3 are upregulated along with ErbB2 (33); however, we found that in normal mouse mammary cells only ErbB3 is upregulated in ErbB2-overexpressing cells whereas EGFR levels remain unchanged. One possible explanation for this difference is that HC11 cells respond differently to ErbB2 overexpression than the human tumor cells used in previous studies.

It is well-established that ErbB2 transcriptionally regulates VEGF (33,40). Using NAFA cells, we determined that ErbB2 regulates VEGF through at least two promoter regions, a distal enhancer corresponding to the promoter sequence from –1.2 kb to –770 bp, and a second regulatory region encompassing the 450 bp proximal and upstream of the transcription start site (+1). Our analysis of the VEGF promoter in ErbB2-overexpressing cells corroborates and expands upon previous studies that showed that ErbB2 leads to an upregulation of VEGF through the distal promoter region, which contains HRE (-985 to -954), a well-known enhancer. Normally, HIF1 protein binds to HRE only under low oxygen conditions, but ErbB2 overexpression indirectly leads to VEGF upregulation by increasing HIF1α synthesis (40). Analysis of a series of promoter deletions revealed that the –160 to –51 bp region of the proximal promoter also mediates ErbB2 induction of VEGF. In addition, this promoter region appears to be responsible for basal VEGF transcription. Therefore, our data suggest that ErbB2 directly regulates VEGF through the core promoter, by increasing basal VEGF transcription.

The VEGF gene does not contain a TATA box or any other recognizable RNA polymerase II binding site. Instead, VEGF appears to be regulated through a GC-rich SP1

binding site (at -80 to –50 bp) (38), a known downstream component of ErbB2 signaling (46). VEGF is regulated through this same promoter region (-80 to –50) by a number of factors including interleukin 1β, hepatocyte growth factor, p42/p44, p53, p73 and von Hippel-Lindau factor (47-52). We found that ErbB2 overexpression regulates VEGF through the core promoter (-80 to –50) by increasing SP1 levels. Although promoter expression of the construct containing mutant SP1 sites (MUT 0.08 kb-luc) is significantly increased over the construct missing both SP1 sites (0.05 kb-luc), we believe the SP1 sites are the main control regions of the core promoter. Perhaps mutation of more than two out of six bases of the SP1 recognition site is required to completely abolish basal expression of VEGF.

VEGF expression is stringently regulated during development and adulthood, as evidenced by both knockout and overexpression studies *in vivo* (2,3,53-55). Consistent with the VEGF gene's dose dependency, organisms have evolved a variety of mechanisms to control VEGF expression. Thus, VEGF regulation appears to be quite complex, occurring at many levels. Relatively little is known about the transcriptional regulation of VEGF, although it has been the focus of many studies since the promoter sequences were cloned (38,56,57). Our data indicate that ErbB2 overexpression regulates VEGF through at least two promoter elements is evidence of the complexity of VEGF gene regulation. In addition, ErbB2 overexpression seems to alter the spatial availability of VEGF accessibility by preferentially increasing VEGF120 production. However, regulation of gene transcription is only one level at which VEGF is controlled. Post-transcriptional control also seems to be important in the regulation of VEGF levels. For example, VEGF mRNA has been shown to have a short half-life of 45 minutes in cells grown under normoxic conditions whereas the half-life is increased to 8 h during hypoxia (58). VEGF isoform production and accessibility are other important points of regulation (9,59).

These mechanisms are likely to be equally as important for VEGF regulation as transcription, but are currently less well understood. Further elucidation of the complex regulation of VEGF could yield future targets for anti-angiogenic cancer treatments that are tumor-specific and pro-angiogenic treatments for ischemia that lead to the formation of more stable vessels.

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Footnotes

1. The abbreviations used are: VEGF, vascular endothelial growth factor; EGF epidermal growth factor; MMTV, mouse mammary tumor virus; DMEM-H, Dulbecco's modified Eagle medium-high glucose; FBS, fetal bovine serum; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; CM, conditioned media; PBS, phosphate-buffered saline; *R*luc, *Renilla* luciferase; Fluc, firefly luciferase; ELISA, enzyme-linked immunosorbent assay; 5' UTR, 5' untranslated region; HRE, hypoxia responsive element; HIF, hypoxia inducible factor.

2. Yin-Shan Ng and Patricia A. D'Amore, unpublished observations.

Figure legends

Figure 1. Effects of EGF and heregulin β1 on VEGF mRNA expression in ErbB2-overexpressing mammary cells. (A) NAFA cells were serum starved for 24 h, treated for 24 hr with EGF (10 ng/ml) or heregulin β1 (100 ng/ml) and then total RNA was collected. (B) VEGF and GAPDH densitometries were first internally normalized to background intensity of blot, then VEGF densitometry was externally normalized to GAPDH densitometry. Ratio of VEGF/GAPDH for 0 h time point was set to "one" and other samples' were graphed relative to the 0 h time point. VEGF was also normalized against ethidium bromide-stained 18S rRNA with similar results (not shown). Experiment was repeated twice with similar results.

Figure 2. Effects of EGF and heregulin β1 on VEGF protein levels in ErbB2-overexpressing mammary cells. Cells were serum starved for 24 h, treated for 48 h with EGF (10 ng/ml) or heregulin β1 (100 ng/ml) and conditioned media and cell lysates were collected. VEGF protein was measured by murine VEGF ELISA in (A) conditioned media and (B) cell-associated lysates. Experiment was repeated twice with similar results.

Figure 3. Overexpression of constitutively active ErbB2 increases endogenous VEGF mRNA levels. HC11 normal mouse mammary cells were transiently transfected with either control vector or constitutively-active ErbB2. Expression of each VEGF mRNA isoform (120,164 and 188) was quantitatively assessed by real time RT-PCR. Value for VEGF188 expression is indicated +/- SD. Experiment was repeated in triplicate twice and one representative experiment is shown. Bars, SD.

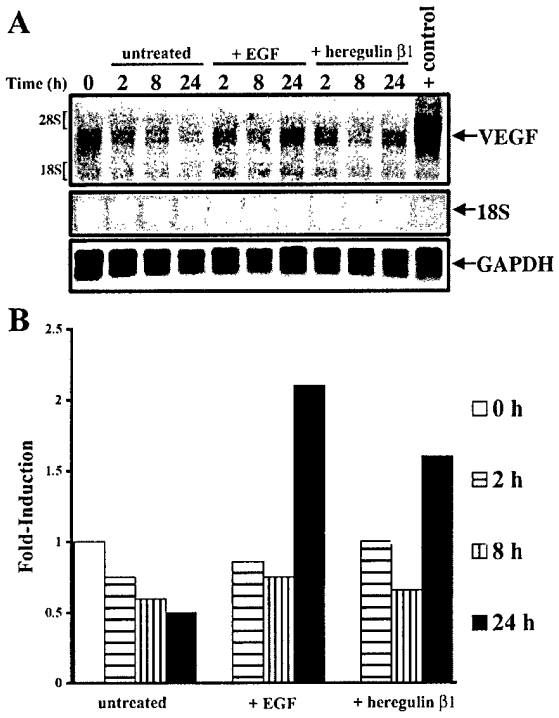
Figure 4. Effect of ErbB2 overexpression on levels of EGFR family members. HC11 normal mouse mammary cells were transiently transfected with control vector, wild type ErbB2 or constitutively-active ErbB2. (A) Antibodies against EGFR, ErbB2 and ErbB3 were used in Western blot analysis of 20 μg of HC11 total protein to analyze changes in EGFR members in response to ErbB2 overexpression. Arrows mark each immunopositive protein: EGFR, 170kDa; ErbB2, 185kDa; ErbB3, 185 kDa. (B) Immunopositive bands were normalized against amido black-stained protein bands (loading control). Experiment was repeated twice with similar results.

Figure 5. Localization of ErbB2 overexpression-sensitive VEGF promoter elements. NAFA cells were transiently transfected with molar equivalents of VEGF promoter luciferase constructs. VEGF/firefly luciferase readings were normalized to cotransfected control *Renilla* luciferase vector. Graph represents fold-change of each VEGF construct promoter activity from the promoterless luciferase construct. This experiment was repeated in triplicate three times and one representative experiment is shown. Bars, SD. *, $P < 0.0001$ compared with promoterless construct.

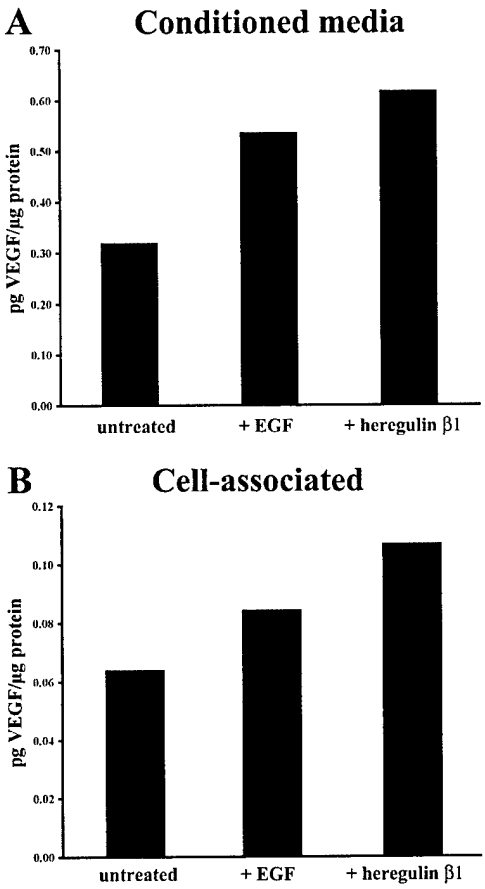
Figure 6. Identification of ErbB2 overexpression-sensitive element within VEGF proximal promoter. VEGF promoter deletions of the 0.45 kb-luc construct were transiently cotransfected into HC11 cells with or without an ErbB2 expression vector. VEGF luciferase values were normalized with cotransfected *Renilla* construct and deletion promoter activity expressed as fold-change from promoterless construct luciferase activity. This experiment was repeated in triplicate three times and one representative experiment is shown. Bars, SD. *, $P < 0.0001$ comparing VEGF-luciferase cotransfected with or without ErbB2 vector.

Figure 7. ErbB2 upregulates VEGF via the SP1 transcription factor. VEGF promoter constructs containing either wild type or mutant SP1 were transiently cotransfected into HC11 cells with or without an ErbB2 expression vector and compared with 0.043 kb-luc and 0.05 kb-luc **(A)** Scematic of VEGF contructs highlighting the sequence differences between wild type and mutant 0.08 kb-luc constructs. SP1 binding site sequences are printed in bold (also underlined in wild type sequence). Mutated bases are printed in bold italics. **(B)** VEGF luciferase values were normalized with cotransfected *Renilla* construct and deletion promoter activity expressed as fold-change from promoterless construct luciferase activity. This experiment was repeated in triplicate three times and one representative experiment is shown. Bars, SD. *, $P < 0.0001$ comparing VEGF-luciferase cotransfected with or without ErbB2 vector. **(C)** HC11 normal mouse mammary cells were transiently transfected with control vector, wild type ErbB2 or constitutively-active ErbB2. Antibody against SP1 was used to immunoprecipitate 500μg of total cell lysate. The same blot containing immunoprecipitated HC11 total protein was probed with two independent antibodies against SP1 the to analyze changes in SP1 levels in response to ErbB2 overexpression. Protein standards were loaded into

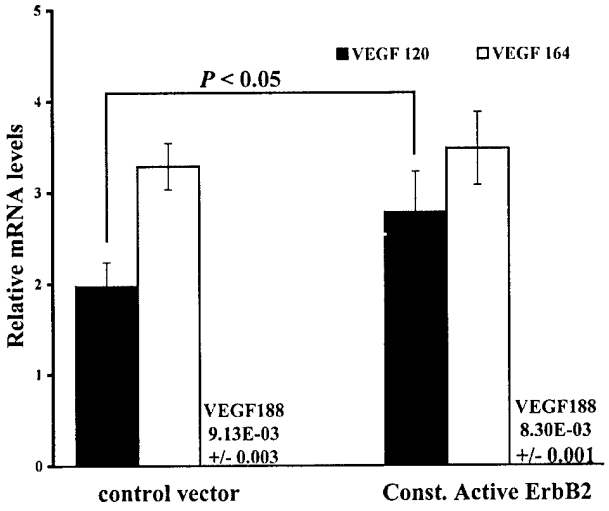
far left lane and 100kDa standard is shown as a reference. Arrows mark immunopositive protein: SP1 is often seen as a doublet of 106kDa and 95kDA. Top antibody predominately recognizes 106kDa band whereas bottom antibody predominately recognizes 95kDa band.



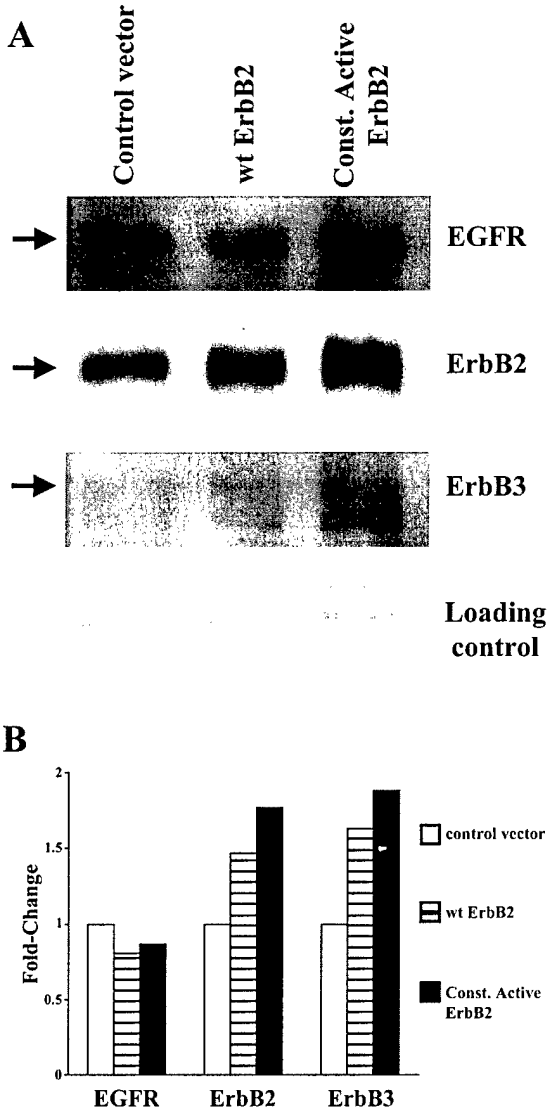
Loureiro et al, Figure 1



Loureiro et al, Figure 2



Loureiro et al, Figure 3



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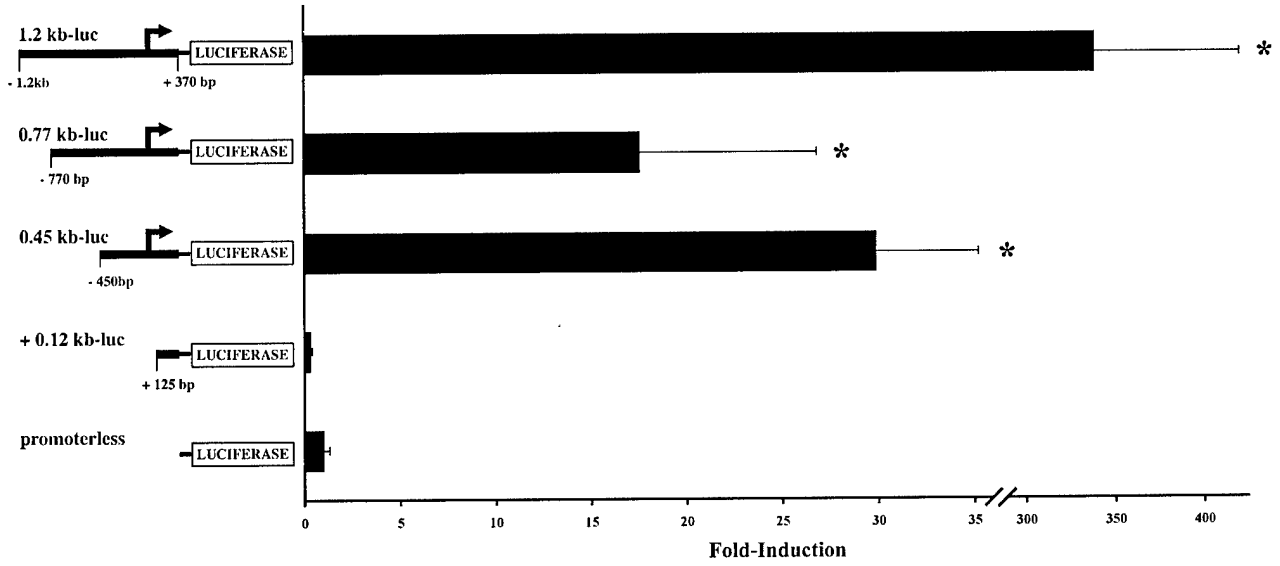
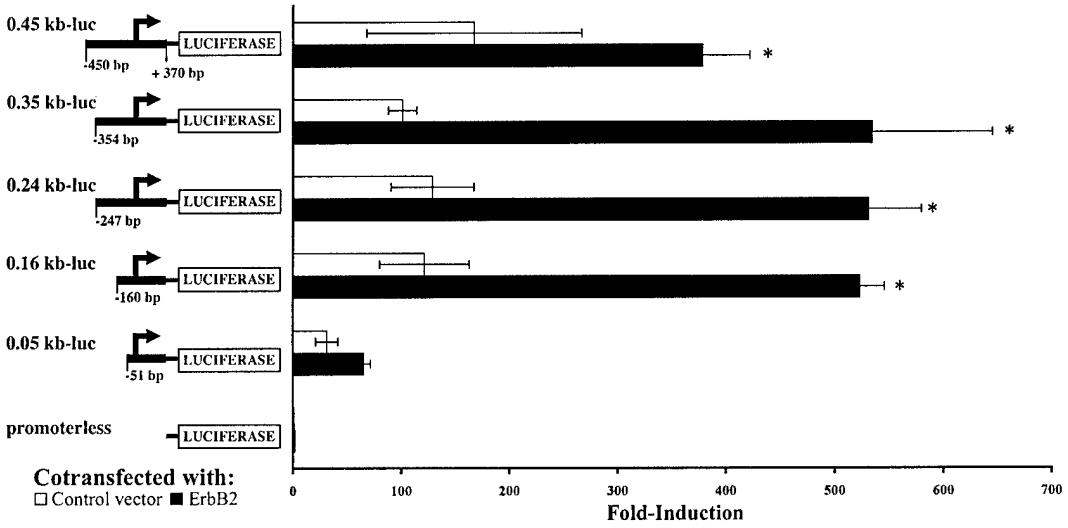
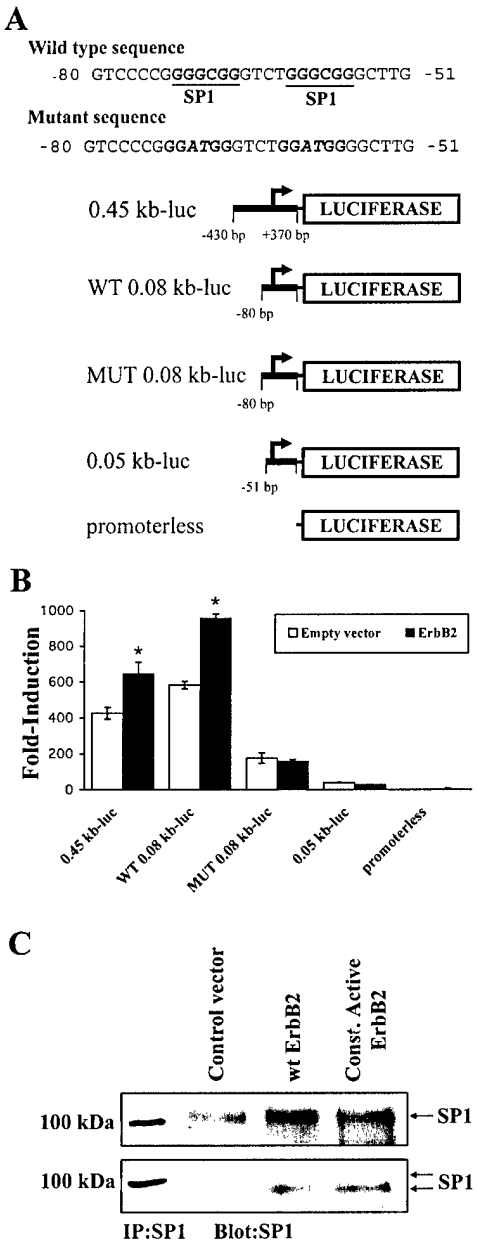


Loureiro et al, Figure 5



Loureiro et al, Figure 6



Loureiro et al, Figure 7